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THE IMMORTAL SENESENCE

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Abstract

Activation of oncogenic signaling paradoxically results in the permanent withdrawal from cell cycle and induction of senescence (oncogene-induced senescence (OIS)). OIS is a fail-safe mechanism used by the cells to prevent uncontrolled tumor-growth and, as such, it is considered as the first barrier against cancer. In order to progress, tumor cells thus need to first overcome the senescent phenotype. Despite the increasing attention gained by OIS in the past 20 years, this field is still rather young due to continuous emergence of novel pathways and processes involved in OIS. Among the many factors contributing to incomplete understanding of OIS are the lack of unequivocal markers for senescence and the complexity of the phenotypes revealed by senescent cells in *in vivo* and *in vitro*. OIS has been shown to play major roles at both the cellular and organismal levels in biological processes ranging from embryonic development to barrier to cancer progression. Here we will briefly outline major advances in methodologies that are being utilized for induction, identification and characterization of molecular processes in cells undergoing oncogene-induced senescence. The full description of such methodologies is provided in the corresponding chapters of the Book.

Introduction

Senescence is defined as an irreversible state of withdrawal from the cell cycle, which can be induced by either physiological signaling (replicative senescence) or aberrant activation of proliferative stimuli (1–5). Despite the lack of active proliferation, senescent cells remain highly metabolically active and are able to influence their environment, thereby modulating both physiological and pathological conditions (6–10).

It is well established that cultured cells have a limited lifespan and can replicate only a determined number of times (the so-called Hayflick limit (11)) before undergoing senescence. Upon activation of the senescent program, cells irreversibly exit the cell cycle and become unresponsive to the action of mitogens. Furthermore, senescent cells undergo morphological and metabolic alterations which lead to enlarged cell and organelles size, senescence-associated β -galactosidase activity, and secretion of extracellular matrix (ECM)-degrading enzymes (12, 13). Many intrinsic cellular factors can contribute to the induction of senescence, which include telomeres shortening, DNA damage, mitochondrial dysfunctions (for comprehensive reviews see Ref (14, 15)), and, more recently, microRNA-driven regulatory mechanisms (16–19). Additionally, a few extrinsic factors have been

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implicated in the establishment/support of the senescent phenotype; these include the matricellular protein CCN1 (also known as CYR61) (20) and other ECM-related components such as integrin β 1 (21) and plasminogen-inhibitor-1 (PAI-1) (22), and secreted factors such as insulin-like growth factor binding proteins (IGFBPs) (23) and interleukin-6 (IL-6) (reviewed in Ref (24)). These observations indicate that senescence is not just dictated by events happening inside the cell but reflects also the integration of cues coming from the cell microenvironment.

Oncogene activation and the resulting aberrant proliferation induce another form of senescence called oncogene-induced senescence (OIS), which is considered one of the first barriers against tumor development (1, 3, 25–28). In many cases, OIS arises once cellular damage is ineffectively dealt with and unrepaired.

OIS Induction

Several cellular models are available to study oncogene-induced senescence, of which the most common is the either constitutive or inducible over-expression of an active form of HRAS (HRAS^{V12}) in human diploid fibroblasts (29–31). With this method, cells become senescent within a week (29) and can be used for investigating senescence markers and phenotypes, as well as the development of screening for the identification of small molecules that can modulate OIS (32).

Intriguingly, OIS can be induced in tumor cells which presumably have already overcome senescence in the course of tumor progression. For instance, depletion of C-MYC to the levels detected in normal melanocytes was found sufficient to induce senescence in several melanoma cell lines (33, 34). Additionally, sustained expression of p21^{WAF1/CIP1} a p53-dependent tumor suppressor gene has been shown to induce senescence in HT1080 fibrosarcoma cells (35). These models carry a high impact as reactivation of OIS in cancer has been recently proposed as a novel mean of therapeutic approach (3, 36–38).

A contentious topic in OIS revolves around the role played by two major tumor suppressors p53 (*TP53*) and p16^{INK4a} (*INK4a/ARF* locus). Studies performed both *in vitro* and in transgenic mice have demonstrated that both proteins actively implement the OIS program in murine systems (39–45). However, their role in OIS in human cells is much less defined and seems to be cell type-dependent. In fact, while p53 depletion is required for the proliferation of human fibroblast expressing constitutive active HRAS (2, 31, 46, 47), it is instead dispensable for senescence induction in human melanocytes (33, 48), keratinocytes (49), and mammary epithelial cells (50). Using primary melanocytes as a model system, it has been recently shown that the RB/p16^{INK4a} pathway regulates cell senescence in part through induction of histone deacetylase 1 (HDAC1)-mediated chromatin remodeling (51) and other studies similarly showed p16^{INK4a} to be essential for RAS-mediated OIS in human cells (52, 53). However, other groups have reported discordant results in which p16 depletion had no effect on RAS (both N-RAS and H-RAS)-induced senescence in human melanocytes (1, 3, 33, 54).

Not only proteins but also micro RNAs (miRNAs) have been widely implicated in the control of OIS. miRNAs comprise a class of fairly recently discovered small non-coding RNAs that have been shown to control gene expression through induction of mRNA degradation or suppression of its translation (55–59). Depending on the targets and context, miRNAs can work as either tumor suppressors or oncogenes, and their expression patterns have been shown to significantly change during physiological and disease conditions, including cancer and senescence (55–59). In recent years, several miRNAs families have been reported to either favor (i.e. the miR17-20a and the miR-106b family (60–62)) or oppose (i.e. miR34a and miR22 (63, 64)) OIS. Some of the mechanisms underlying these effects include suppression of the cell cycle inhibitor p21^{WAF1/CIP1} (60, 62) and suppression of the C-MYC oncogene (63). Additionally, miRNAs have been shown to downregulate other important cell cycle promoters such as SIRT1 (a direct modulator of the p16-Rb and p53 pathways (65–67)), Sp1 (a transcription factor regulating the expression of p53 and many other genes involved in cell cycle (68, 69)), and CDK6 (which phosphorylates pRb to delay senescence (70, 71)) (64).

Additionally, a novel class of small non-coding RNAs called circularRNAs (cirRNAs) has been recently identified. CircRNAs functions are not well understood, however it has been shown that they can interact with several molecules of miRNA at a time, acting like “sponges” to reduce miRNA availability (72–75). The use of genome-wide miRNA and circRNA screenings emerges as an important tool for the identification of additional players involved in either the establishment of oncogene-induced senescence or facilitating its bypass (60, 76–78).

Metabolic Changes Detected During OIS

While the definition of OIS is well established, its phenotypical characterization suffers from the lack of unambiguous markers (79–81). Therefore, OIS detection necessitates the use of a combinatorial approach with multiple markers, highlighting the need for improved methodologies (80).

One of the most classical senescence detection assays is based on the activation status of senescence-associated β -galactosidase (SA- β -gal), an enzyme that normally resides in the lysosomes and is up-regulated in senescent cells. SA- β -gal activity is detected at sub-optimal pH (pH 6.0) using either a chromogenic (5-bromo-4-chloro-3-indolyl β -D-galactopyranoside, X-Gal) (12) or a fluorescent substrate (fluorescein-di-D-galactopyranoside, FDG) (82). However, SA- β -gal activity can be influenced by a plethora of other stimuli and therefore displays a high frequency of false positive results (12, 80, 83, 84). Moreover, while SA- β -gal staining can be performed on frozen samples, it cannot be used on fixed samples, thereby limiting its applicability *in vivo* (80). To this end, an improved Sudan Black B (SBB) histochemical stain has been recently described for detection of lipofuscin (an auto-fluorescent aggregate of oxidized proteins often found in both aged and senescent tissues (85, 86)). In a parallel comparison with SA- β -gal staining, the improved SBB has shown promising results for the accurate detection of senescent cells in culture, as well as it revealed superior ability to detect senescent cells in tissue samples, including paraffin-embedded materials, extending its applicability (87).

Another well-characterized aspect of senescence is the secretion of a distinct subset of cytokines and factors, collectively named the senescence-associated secretory phenotype (SASP) (88). The SASP has been shown to exert paracrine interactions to modulate the reinforcement and/or propagation of the senescent status (8–10, 89). Some of the key players which are induced by and in turn sustain and propagate the senescence phenotype belong to the family of the interleukins (especially the pro-inflammatory IL6 and IL1, as well as IL8) (8–10, 89, 90). In addition, components of the tumor growth factor (TGF)- β and insulin-like growth factor (IGF)/IGF receptor pathways have shown to play a prominent role in the SASP (8–10, 89, 90). However, it is important to note that the full composition and effectors of the SASP is strongly influenced by the type of model system used (6). Additionally, depending on the cellular context, the SASP has been shown to have either pro-tumorigenic or tumor suppressor functions (7). Classically, the SASP is identified through ELISA or qRT-PCR assay for some of its major components; however, more recently a novel approach based on widefield high-content microscopy has been reported (90). This method allows for automatic acquisition and quantitative analysis of SASP makers in a 96-well format which is suitable for development of high-throughput systems for the identification of SASP-(and therefore OIS-) modifying agents.

DNA damage is one of the main inducers of senescence. In the context of OIS the DNA damage was believed to be caused mainly by reactive oxygen species (ROS) induction (91, 92) and the hyper-replication of genomic DNA i.e. multiple firing of the same replication origin (47). Another source of DNA damage in cells undergoing OIS originates from dysfunctional telomeres. Although telomere erosion is classically associated with replicative senescence, recent studies have shown that OIS can result in dysfunctional telomeres associated with DNA damage (telomere dysfunction-induced DNA damage foci, TIF) (93). TIF elicit the same DNA damage response (DDR) as non-telomeric lesions; however, while non-telomeric DDR foci get repaired over time, TIF are persistent and have been detected *in vivo* in pre-malignant lesions (93–95).

Recently, we and others highlighted a novel mechanism by which DNA damage is induced in cells undergoing OIS. It has been shown that activated HRAS signaling suppresses levels of key deoxyribonucleoside biosynthesis enzymes including thymidylate synthase (TS) and subunits of ribonucleotide reductase (RRM1 and RRM2) (96, 97). This results in depletion of cellular dNTP pools which in conjunction with HRAS-induced DNA polymerase activity results in severe DNA damage (96, 97). Interestingly, TS, RRM1 and RRM2 have been verified as *bona fide* targets of C-MYC (96, 98–100). Consistently, ectopic expression of C-MYC has been shown to increase the intracellular nucleotide pools (99–101), and to suppress oncogene-induced senescence in normal and transformed human melanocytic cells (33, 98). In support of the role of nucleotide levels in control of OIS, it has been shown that supplementation with deoxyribonucleotides or ectopic expression of enzymes involved in their biosynthesis (TS, RRM1, RRM2) was sufficient to bypass the senescent phenotype induced by either overexpression of oncogenic RAS (H-RAS) in normal cells (96, 97) or by depletion of C-MYC in melanoma cells (98). Therefore, intracellular dNTP levels emerge as important modulators of DNA damage and OIS in normal and transformed cells.

The changes described above are just a fraction of a larger scale metabolic alterations occurring in cells undergoing OIS, and the global metabolic changes occurring during oncogene-induced senescence have been the focus of study of several groups (102–106). Some of the other pathways altered during OIS include the oxidation of fatty acids (103), glucose metabolism (6), mitochondrial oxygen consumption (103), as well as protein ubiquitination (106).

OIS-undergoing cells present with a distinct signature of metabolites compared to cells that experienced replicative senescence, including decreased lipid synthesis as well as increased fatty acid oxidation due to increased levels of inactive acetyl-CoA carboxylase 1 (ACC1) (103). Cells undergoing OIS also display a high basal rate of oxygen consumption, which is a major reason for the above-mentioned increase in fatty acid oxidation concomitant with no increase in mitochondrial uncoupling (103).

Ubiquitination is a common post-translational modification (PTM), which can either direct proteins for degradation through the 26S proteasome system (polyubiquitination) or can alter a protein function (monoubiquitination) (107, 108). The process of ubiquitination is highly dynamic, being regulated by both ubiquitin ligases (E1, E2, and E3 enzymes) which add ubiquitin moieties to proteins, and deubiquitinating enzymes (DUBs) which instead remove the tag (109). A recent paper profiled the changes in protein ubiquitination patterns occurring during OIS and identified most of the alterations being clustered within the mammalian target of rapamycin (mTOR) downstream effectors pathways: 4EBP-EIF4E, p70S6K and EEF2K/EIF2 (106). These pathway plays a prominent role in the translational control of cell growth and proliferation (110).

mTOR is also critical for the regulation of autophagy, a tightly controlled cellular program of self-degradation which is activated in response of several stress in order to maintain an energetic balance (110–116). Autophagy is characterized by the formation of double-membrane vesicles (autophagosomes) which deliver unwanted or damaged cellular material to the lysosome for degradation (111). It has been established that autophagy is activated during OIS (115–117); however, its role in the senescent phenotype is far from fully elucidated. Recent papers have demonstrated that autophagy is induced by, and at the same time contributes to the establishment of OIS through induction of the SASP *via* mTOR activation (TOR-autophagy spatial coupling compartment, TASCC) (116, 117). At the same time, autophagy inhibition has been suggested to promote senescence in certain settings (118). A recent study reconciled these findings unveiling differential behaviors of selective autophagy and general autophagy toward senescence (119). Selective autophagy is a process by which cells selectively degrade certain molecules via interaction with specific adaptors, one of which is p62 (120–122). p62 was shown to target the transcription factor GATA4 (a member of the zinc-finger family of transcription factors (123)) for degradation (119). GATA4 has been implicated in the induction of the SASP through positive regulation of NF- κ B, one of the major regulators of cytokines production (119). Thus, selective autophagy may act as a senescence suppressor by downregulating senescence effectors (such as GATA4). However, senescence stimuli allow for escape of GATA4 from p62-mediated degradation and help establishing the process of general autophagy, which is a positive contributor to senescence.

Detection of Senescence *in vivo*

Most of the analyses described so far have been performed mainly in cultured cells. Studying OIS *in vivo* is hindered by many factors, including heterogeneity in responses to oncogene activation in different tissues, expression of senescence-associated markers in *non bona fide* senescent cells, and limited efficacy of reagents. However, several reports described OIS *in vivo*.

In humans, the most natural example of OIS is represented by nevi, benign aggregations of melanocytes that exited the cell cycle (1, 3, 54, 124, 125). A high proportion of melanocytes in nevi harbor activated BRAF^{V600E} or NRAS^{Q61R} proteins. Surprisingly, the same mutations have been found in malignant melanomas often at lower frequencies, suggesting that suppression of OIS is a prerequisite for tumor progression (126, 127). Human melanocytic nevi display several hallmark of OIS, including cell cycle arrest (assessed by absence of Ki-67 staining, a marker of cell proliferation) and increased SA- β -gal activity (54). At the same time, when stained for telomere FISH, nevomelanocytes do not display signs of telomere erosion or loss (which is an indication of age-related senescence) (54).

Transgenic mouse models for tumor initiation are also available, in which the oncogenic K-rasV12 allele expression is induced by Cre recombinase in restricted tissues. Using lung- or pancreas-specific systems, researchers were able to visualize senescence in premalignant tumors using SA- β -Gal staining and BrdU incorporation, as well as with antibodies toward OIS effectors (including p16^{INK4a} and p15^{INK4b}) (128, 129).

Lower organisms such as Zebrafish (*Danio rerio*) and Drosophila have been used as well for studying OIS. In Zebrafish, expression of a heat shock-inducible human HRAS^{V12} was shown to result in robust accumulation of ROS (130). ROS induction was mediated by two orthologs of Nox4 (which is essential for ROS induction by RAS in human cells) (130). Additionally, conditional expression of human HRAS^{V12} induced DNA damage response (DDR) and cell arrest in a *tp53*-dependent fashion (131). In Drosophila instead, active Ras required concomitant induction of mitochondrial dysfunction in order to fully induce a senescent phenotype. The combination of HRas^{V12} and mitochondrial dysfunction was necessary to induce oxidative stress and activate c-Jun amino (N)-terminal kinase (JNK) signaling. Ras and JNK together suppressed the Hippo pathway and induced senescence (132).

Another form of senescence highly reminiscent of OIS is the therapy-induced senescence (TIS). TIS is often a consequence of anti-cancer therapy and has been shown to be induced in both tumor cells lines and in patients (38, 133–141). TIS and OIS share several downstream effectors and phenotypes as they both evoke a DDR. However, DNA damage is generated with different modality of actions: oncogenic induction of DNA damage arises from dNTPs depletion, ROS production and multiple firing from the same origin of replication (as described above) (34, 47, 91, 92, 96–98, 100); TIS-induced DNA damage is instead a result (direct or indirect) of the therapeutic agent in use, although sometimes the modality may overlap with OIS as, for example, some therapeutic agents act *via* depletion of nucleotide pools (142).

Because of its cytostatic effects TIS has recently been proposed as a new strategy for cancer therapy (38, 133–141, 143). At the same time, long-term persisting tumor senescent cells can profoundly alter the microenvironment through SASP-mediated paracrine effects and detrimentally affect neighboring cells (8–10, 88, 89, 113). In fact, it has been shown both *in vitro* and *in vivo* that factors from the SASP exacerbate malignant growth and behavior of tumor cells from several malignancies, including breast and prostate cancer as well as melanoma (88).

One of the best characterized systems for the study of TIS is a primary murine Myc-driven lymphoma model. In this model, cells have been engineered to stably over-express Bcl2 to prevent apoptosis and obtain a homogenous TIS response (38, 137). This allows for monitoring the effects of various genetic alterations on TIS establishment and downstream effects (38, 136, 137, 140, 141, 144), including knockout of *p53* or *p16^{INK4a}*, inactivation of DDR, and alteration of SASP factors (i.e. NF- κ B and TGF- β).

Using the mouse model described above combined with treatment with cyclophosphamide (CTX) it has been shown that elimination of TIS lymphoma cells *in vivo* resulted in improved outcome, highlighting the harmful effects of long-lasting tumor senescent cells on the organism (141). TIS cells were found to have a strongly enhanced glucose uptake and ATP production through glycolytic activity, reinforcing the Warburg effect (141), and this phenomenon was linked to the high proteotoxic stress induced by the SASP (88, 145). At the same time, this increased glucose demand made TIS cells more sensitive to glucose uptake blockage and autophagy induction, which resulted in their caspase-dependent apoptosis, followed by tumor regression and longer-lasting therapeutic effects (141).

Finally, although senescence was first characterized in the context of aging and tumor suppression, it has been recently discovered that senescence contributes to embryonic development and tissue repair (20, 146–149). Mouse embryos were found to express several markers and mediators of senescence, including SA- β -gal activity and H3K9me3 (146, 147). Interestingly, the developmental senescence and OIS share a molecular signature which include senescence inducers p21^{WAF1-CIP1} and p15, as well as SASP regulators (such as *CEBPB*, *IGFBP5*, *WNT5a*, and the TGF- β -pathway) (146, 147).

Senescence has been shown to be activated also during wounding and pathological conditions to promote healing. Cutaneous wounds induce a rapid senescence response in fibroblasts and endothelial cells and mediate release of platelet-derived growth factor AA (PDGF-AA) as part of the SASP (148). PDGF-AA induces myofibroblast differentiation to promote an efficient wound closure (148). During hepatic fibrosis, stellate cells that become senescent are more efficiently cleared by natural killer cells to limit the tissue damage (149).

Concluding Remarks and Future Perspective

The molecular processes occurring in cells undergoing oncogene-induced senescence appear to overlap with those of replicative, developmental, as well as therapy-induced senescence. While it is well appreciated that some of these same mechanisms may also contribute to tumor initiation and escape from therapy-induced death, more work needs to be done toward

understanding which pathways and which components are responsible for it. To this end, improved methods for detection of OIS and its associated phenotypes are crucially needed. In the long run, this knowledge will potentially lead to the development of better therapeutic approaches and result in long-lasting response and increased survival of patients.

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